Transforming Growth Factor β1 Induces Apoptosis in Normal Melanocytes but not in Nevus Cells Grown in Type I Collagen Gel

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We used type I collagen gel cultures to compare the growth requirements of melanocytes and dermal nevus cells. Melanocytes but not nevus cells undergo apoptosis in collagen unless supplied with growth stimulators such as fibroblast growth factor 2. To characterize the mechanism of melanocyte apoptosis in collagen, we tested the effects of transforming growth factor β1, known to be functionally active in the skin. When picomolar amounts of transforming growth factor β1 were added to normal melanocytes grown in type I collagen gel, their apoptosis was dramatically accelerated. In contrast, the apoptotic rate of nevus cells and melanoma cells grown under similar conditions was not affected by transforming growth factor β1. The increased apoptosis of normal melanocytes was effectively counteracted by addition of either neutralizing transforming growth factor β1 antibodies or fibroblast growth factor 2 to the collagen gel. Interestingly, the background apoptosis of normal melanocytes was also inhibited by transforming growth factor β1 antibodies. By Western blotting we detected transforming growth factor β-like immunoreactivity in melanocyte, nevus cell, and melanoma cell lysates. A sensitive bioassay confirmed that their medium contained considerable amounts of heat-activatable growth inhibitory activity that could partly be neutralized by transforming growth factor β1 antibodies. It is evident that apoptosis of melanocytes grown in type I collagen gel can be mediated by both endogenous and exogenous transforming growth factor β. We suggest that the balance between inhibitory growth factors such as transforming growth factor β and stimulatory growth factors like fibroblast growth factor 2 has the potential to regulate the growth, localization, and survival of normal melanocytes also *in vivo*. The resistance of nevus cells to transforming-growth-factor-β-mediated apoptosis may facilitate their ability to grow in the dermal compartment of the skin. ***Key words: extracellular matrix/FGF-2/growth inhibition/melanoma/TGF-β. J Invest Dermatol 115:286–291, 2000***

Melanocytes grow restricted to a defined locus in the mature organism, tightly controlled by their growth factor requirements and, as it seems, by their dependence on certain matrix components (Halaban, 1996; Scott *et al*, 1997). Nevus cells are in many ways similar to normal melanocytes but they are able to enter the dermal skin compartment. We have previously shown that when melanocytes are grown *in vitro* in a three-dimensional collagen lattice they undergo apoptosis unless stimulated by survival factors, like fibroblast growth factor 2 (FGF-2) provided *in vivo* to epidermal melanocytes by adjacent keratinocytes (Halaban *et al*, 1988). In contrast, nevus cells that synthesize substantial amounts of FGF-2 survive and even proliferate in collagen gel (Alanko *et al*, 1999).

Transforming growth factor βs (TGF-βs) comprise a family of multifunctional polypeptides (see Bonewald, 1999, for a recent review). Three different subtypes, TGF-β1 through TGF-β3, have been described in mammals with similar biologic activities (Taipale *et al*, 1994). TGF-β has extremely variable effects on different cell types. In the dermis TGF-β enhances matrix deposition by the connective tissue cells and induces angiogenesis during wound healing (Roberts *et al*, 1986; Massagué, 1990). In the epidermis it functions as a growth inhibitor helping to maintain tissue homeostasis (Wang *et al*, 1997; 1999). The presence of TGF-β1 in the dermal connective tissue (Karonen *et al*, 1997) led us to test its effects on melanocytes and nevus cells.

TGF-β is secreted from cells as a latent precursor molecule. Soon after secretion the precursor is proteolytically cleaved to produce mature TGF-β that is inactive as it still remains noncovalently bound to the latency-associated peptide (Gentry *et al*, 1988). This latent TGF-β complex is bound to microfibrillar structures of the extracellular matrix (ECM), where it can be released and activated by proteolysis (Taipale *et al*, 1994). ECM-resident growth factors, such as TGF-β and FGF-2, are important mediators of cell differentiation, growth, and survival. Immobilization and storage of growth factors in the ECM as either active or latent molecules helps to focus and localize their effects in the immediate microenvironment of the synthesizing cells. Activated TGF-β exerts its biologic effects by forming a complex with its cell surface binding protein betaglycan, which facilitates stimulation of the two types of transmembrane TGF-β receptors (Wrana *et al*, 1994).

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Abbreviations: CT, cholera toxin; TUNEL, TdT-mediated dUTP-FITC nick end labeling.

TGF-β has recently been shown to induce apoptosis in several cell types (Lafon *et al*, 1996; Islam *et al*, 1997; Yan and Sage, 1998). On the other hand, TGF-β1 protects macrophages from serum-deprivation-induced apoptosis (Chin *et al*, 1999), and overexpression of TGF-β protects lymphocytes against tumor necrosis factor cytotoxicity and causes immune defects and even malignancies. In a recent paper the lymphoprotective effect of TGF-β was attributed to induction of an antiapoptotic adhesion protein (Carey and Chang, 1998).

In this work we have studied the mechanisms underlying melanocyte death in collagen. We found that melanocytes grown in three-dimensional type I collagen become exceptionally sensitive to endogenous or exogenous TGF-β, whereas both dermal nevus cells and melanoma cells from metastatic melanomas are resistant to the apoptotic effect of TGF-β.

MATERIALS AND METHODS

Cell culture Human skin melanocyte cultures were established as described by Alanko *et al* (1999). Briefly, the dermis of a skin biopsy was mechanically removed. The epidermis was separated from the rest of the dermis with dispase (Collaborative Biomedical Products, Bedford, MA) treatment. The isolated epidermis was then treated with trypsin-ethylenediamine tetraacetic acid to produce a cell suspension consisting mainly of keratinocytes and melanocytes. The cells were cultivated in a selective F-12 medium supplemented with 10% fetal bovine serum (FBS; Life Technologies/Gibco BRL), 1 μg per ml cholera toxin (CT; Sigma, St. Louis, MO), 10 ng per ml phorbol 12-myristate 13-acetate (PMA; Sigma), and 3 ng per ml human recombinant FGF-2 (a gift from Dr. Andreas Sommer, Synergen, Boulder, CO). After the plates had reached about 50% confluence they were treated with Geneticin (antibiotic G418, 100 μg per ml; Sigma) for 7–10 d to kill remaining fibroblasts and keratinocytes.

Nevus melanocyte cultures were also initiated as described by Alanko *et al* (1999). A 3 mm biopsy was obtained from an excised intradermal or compound-type nevus. The epidermis was mechanically removed to the depth of the dermal papillary layer. The remaining dermis was cut into pieces and collagenase (type XI, Sigma) was used to produce a single-cell suspension. The cells were plated and treated like normal melanocytes (above).

After Geneticin treatment 100% of the cells stained positive for S-100 (antibodies from Dako, Denmark) and GD3 ganglioside (antibodies from Biogenesis, Bournemouth, U.K.) with no detectable contamination by keratinocytes or fibroblasts. Four normal melanocyte lines and two nevus cell lines were used between passages 4 and 12.

Before every experiment the medium was changed to F-12 medium containing 10% FBS without CT, PMA, and FGF-2 for 24 h and the experiments were carried out in the absence of these factors unless stated otherwise.

Melanoma cell line ML793 (also designated WML793 or WM793) has been established at the Wistar Institute from a vertical growth phase melanoma primary tumor (Herlyn *et al*, 1985). Line G-361 was from American Type Culture Collection. They were both cultured in RPMI-1640 medium supplemented with 10% FBS.

Mink lung epithelial (Mv1Lu) cells were from American Type Culture Collection. They were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS.

Construction of collagen lattices To produce a three-dimensional collagen gel, acid-soluble rat tail collagen (type I collagen, 3–4 mg per ml; Upstate Biotechnology, Lake Placid, NY) was mixed 1:1 with 2 × medium. The final concentration of FBS was 10% unless stated otherwise. The gel was allowed to polymerize for 30 min at 37°C. Cells were seeded on top of the first collagen layer at 25,000–50,000 cells per cm^2. They attached tightly in 30 min. Unattached cells (<5%) were rinsed away and a second layer of collagen gel was cast. After solidification of the second layer, medium was added on top to provide the cells with sufficient nutrients during prolonged experiments. FGF-2, human recombinant TGF-β1 (R&D Systems, Minneapolis, MN), human recombinant tumor necrosis factor α (TNF-α) (Boehringer Mannheim, Mannheim, Germany), human interferon-α (IFN-α) (highly purified human leukocyte IFN-α, kindly provided by Dr. Hannele Tölö, Finnish Red Cross Blood Transfusion Service, Helsinki, Finland), or neutralizing chicken egg yolk anti-TGF-β1 IgY (R&D Systems) was added as indicated. Similar results were obtained when the factors of interest were either included in the collagen gel or added to the growth medium from which most substances readily spread to the collagen layers by passive diffusion.

TUNEL staining Apoptotic DNA cleavage was detected *in situ* by a slight modification of the original TUNEL method (TdT-mediated dUTP-fluorescein isothiocyanate (FITC) nick end labeling) (Gavrieli *et al*, 1992). The collagen gels were fixed overnight with 3.7% formaldehyde in phosphate-buffered saline (PBS). The gels were washed several times with water and covered with a labeling mixture containing 1 nM fluorescein-12-dUTP (NEN Life Science Products, Boston, MA), dATP (Boehringer Mannheim), 0.2 U per μl terminal deoxynucleotidyl transferase (TdT; Promega, Madison, WI) and 1 × TdT buffer (supplied with the enzyme) in water. After 4 h in 37°C the reaction was stopped by immersing the specimens in 2 × sodium citrate/chloride buffer. The nuclei were briefly stained with 1 μg per ml bisbenzimide (Hoechst 33342, Sigma) in PBS. After extensive washing in PBS the gels were mounted in PBS:glycerol (1:1) and examined in a Leitz fluorescence microscope equipped with the appropriate filters. Hoechst stained nuclei (300–500) were evaluated for FITC fluorescence (TUNEL) and morphology (Hoechst staining).

5-Bromo 2′-deoxyuridine (5-BrdU) incorporation and immunostaining Cell proliferation was quantitated *in vitro* by 5-BrdU incorporation and immunostaining. 5-BrdU incorporates to nuclear chromatin during replicative DNA synthesis. Briefly, the cells were seeded on glass coverslips and grown overnight. The factors of interest were added to the growth medium and the incubation was continued for 48 h; 250 μM 5-BrdU (Sigma) was added 4 h (melanoma cells) to 48 h (melanocytes) before the end of the incubation. The cells were fixed for 5 min with ice-cold 100% methanol. Nuclei were permeabilized by brief treatment with 1.5 N HCl. The cells were stained with monoclonal anti-BrdU antibodies (Amersham) and a secondary rhodamine-conjugated antibody (Jackson). The nuclei were briefly stained with Hoechst as above. Hoechst stained nuclei (300–500) were evaluated for rhodamine fluorescence.

Western blotting Cells were lyzed with 0.5% NP-40 in 120 mM NaCl, 25 mM Tris, pH 8, in the presence of protease inhibitors. The lysates were centrifuged and an aliquot of the supernatant was taken for protein measurement. Equal amounts of protein were mixed with 2 × reducing Laemmli sample buffer, boiled, and electrophoresed in a 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Conditioned media were collected after 24 h incubation. Media were concentrated 10 × with Centricon 10 (Amicon, Beverly, MA). A 50 μl aliquot was mixed with 2 × reducing Laemmli sample buffer and electrophoresed as above. Collagen solution was mixed with 2 × reducing Laemmli sample buffer and electrophoresed as above.

After electrophoresis the proteins were electroblotted onto a nitrocellulose filter (Schleicher & Schuell, Keene, NH) and TGF-β was detected by rabbit anti-TGF-β1 antibodies (Taipale *et al*, 1992) (a gift from Dr. Jorma Keski-Oja, University of Helsinki, Finland) followed by biotin-conjugated goat antirabbit antibodies and streptavidin-HRP (Dako). HRP was visualized with enhanced chemiluminescence (ECL) following the manufacturer's instructions (Amersham). Human recombinant TGF-β1 (R&D Systems) was used as a positive control.

Mv1Lu growth inhibition test The growth-inhibitory activity secreted by cultured cells was analyzed in Mv1Lu cell assay (Laiho *et al*, 1990). Mv1Lu cells are very sensitive to growth inhibition by TGF-β. Briefly, Mv1Lu cells were plated on 24 well dishes 24 h prior to the experiment. The conditioned medium of interest was clarified by centrifugation. Mv1Lu medium was replaced by the conditioned medium. After 18 h 5-BrdU was added for 2 h. The cells were fixed with 3.5% paraformaldehyde and permeabilized with 0.5% NP-40 and 1.5 N HCl, and the incorporation of 5-BrdU was measured essentially as described above. The growth inhibitory activity was identified as TGF-β by using neutralizing chicken egg yolk anti-TGF-β1 IgY (R&D Systems) antibodies. Normal chicken IgY (1 μg per ml; R&D Systems) and preimmune rabbit IgG (1 μg per ml) were tested as negative controls. When indicated, the conditioned medium was heat treated (90°C, 1 min) before transfer to Mv1Lu cells to activate latent forms of TGF-β (Brown *et al*, 1990). Serial dilutions of human recombinant TGF-β1 were used as standards.

RESULTS

TGF-β1 inhibits the growth of cultured melanocytes and nevus cells Normal human skin melanocytes need substantial protein kinase C and cAMP stimulation to proliferate *in vitro*. When cultivated in the absence of stimulatory factors they cease to divide and slowly begin to deteriorate (Alanko *et al*, 1999). The cells probably gradually undergo apoptosis but this happens very

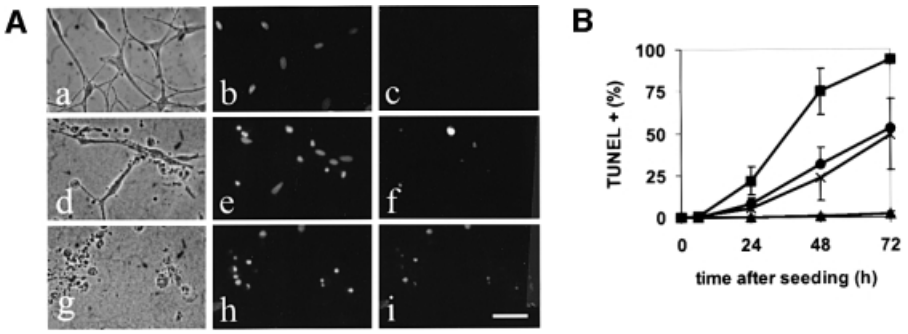

Figure 1. TGF-β1 enhances apoptosis in melanocytes in collagen gel. Human skin melanocyte cultures were fixed with PFA after 0, 6, 24, 48, or 72 h of cultivation on a conventional cell culture plate or in collagen gel. Cells were stained with bisbenzimide (Hoechst 33342) and by TUNEL. (*A*) The phase contrast (*a*, *d*, *g*), Hoechst stained (*b*, *e*, *h*), and TUNEL stained (*c*, *f*, *i*) photomicrographs after 48 h incubation are shown. The cells growing on conventional culture plate stayed spindle-shaped (*a*) and had evenly stained nuclei (*b*) that were negative in TUNEL staining (*c*). In collagen almost 50% of the cells fragmented (*d*), had nuclear fragmentation typical for apoptosis (*e*), and stained positive in TUNEL (*f*). After 48 h in the presence of 1 ng per ml TGF-β1 the great majority of the cells were fragmented (*g*), showing nuclear fragmentation (*h*) and TUNEL staining (*i*). *Scale bar* (*i*): 25 μm. (*B*) 300–500 nuclei were evaluated for TUNEL staining after cultivation in collagen for 6–72 h in the absence (control, ●) and presence of FGF-2 (5 ng per ml, ▲), TGF-β1 (1 ng per ml, ■), or both FGF-2 and TGF-β1 (×). The percentage of positive (apoptotic) cells is shown (*error bars*: ± SD in three experiments with different melanocyte lines).

slowly and the apoptotic cells detach from the monolayer. By TUNEL staining apoptotic cells can rarely be detected (**Fig 1*A***). We wanted to test whether TGF-β1 has an effect on melanocyte survival or growth. We did not observe increased detachment or TUNEL staining in monolayers of melanocytes in the presence of TGF-β (data not shown). Next, BrdU labeling of the cells was carried out to measure their replicative activity. Unstimulated melanocytes are quiescent and TGF-β1 did not have an effect on them. In contrast, TGF-β1 effectively inhibited the replication of melanocytes stimulated by CT, PMA, and FGF-2 (**Fig 2**). We also wanted to test whether TGF-β1 could affect the growth of other cell types of the melanocytic lineage. The cells tested were dermal nevus cells and two phenotypically different melanoma cell lines. Unlike normal melanocytes, nevus cells proliferate slowly also in the absence of stimulants. Their growth was sensitive to inhibition by TGF-β1. ML793 and G-361 melanoma cell lines were both totally unresponsive (data shown only for ML793; **Fig 2**).

In collagen gel cultures the apoptosis of normal melanocytes is greatly accelerated by small amounts of TGF-β Collagen gel was used as a three-dimensional growth environment. When normal melanocytes were grown in the absence of stimulatory factors between two gel layers (or on top of a single layer; data not shown), the proportion of TUNEL positive cells increased rapidly with time. When TGF-β was added to collagen cultures it caused a significant acceleration of apoptosis (**Fig 1*A***, ***B***). FGF-2, shown to promote the survival of melanocytes in collagen gel (Alanko *et al*, 1999), could effectively counteract the TGF-β effect (**Fig 1*B***). The TGF-β effect was dose dependent and detectable already at concentrations as low as 0.1 ng per ml (≈ 4 pmol per l) (**Fig 3**). In contrast, TGF-β did not induce apoptosis in nevus cells or melanoma cells grown in collagen gel (**Fig 4**). To demonstrate specificity of the phenomenon melanocytes were cultured in collagen gel either with TGF-β1 alone or with TGF-β1 preincubated with antibodies. Preincubation with the antibodies together with 1 μg per ml anti-TGF-β1 included in the gel and administered daily could almost completely inhibit the effect of 0.5 ng per ml TGF-β1 (**Fig 5**). We also tested the effect of two other growth inhibitory factors with apoptotic potential, namely TNF-α and IFN-α, on melanocyte apoptosis. Fifty nanograms per milliliter TNF-α or 1000 IU per ml IFN-α did not accelerate the apoptosis of melanocytes in collagen gel (data not shown).

TGF-β antibodies can reduce also the "background" apoptosis of normal melanocytes grown in collagen When neutralizing TGF-β1 antibodies alone were added to melanocytes grown in collagen, their apoptotic rate diminished. One microgram per milliliter anti-TGF-β1 included in the gel and

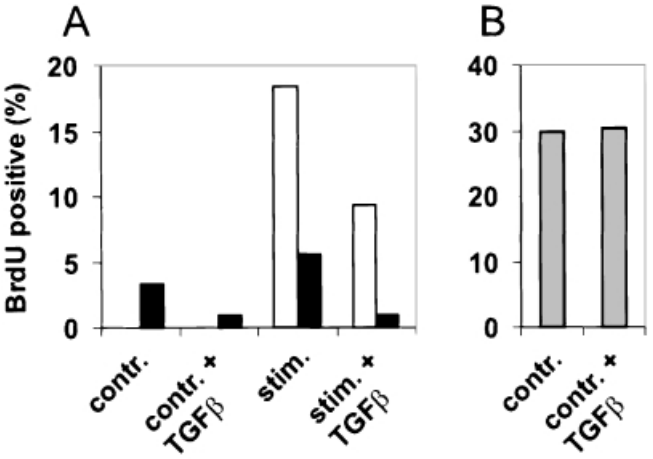

Figure 2. TGF-β1 inhibits the replication of nevus cells and stimulated melanocytes but has no effect on quiescent melanocytes or melanoma cells. Melanocytes (*open bars*), nevus cells (*black bars*), and melanoma cells (*gray bars*) were seeded on a conventional cell culture plate in F-12 + 10% FBS. After overnight incubation the medium was changed and, where indicated, CT (1 μg per ml), PMA (10 ng per ml), and FGF-2 (3 ng per ml; stim.), and/or TGF-β1 (0.5 ng per ml), were added. Incubation was continued for 48 h and 250 μM 5-BrdU was added 48 h (melanocytes and nevus cells, *A*) or 4 h (melanoma cells, *B*) before the end of the incubation period. The cells were stained with monoclonal anti-BrdU antibodies and bisbenzimide. The percentage of 5-BrdU positive cells is shown.

added daily caused a consistent 20%–50% reduction in the proportion of TUNEL-positive apoptotic cells after 2 d (**Fig 5**). Higher antibody concentrations failed to make the effect more evident. TGF-β1 antibodies had no effect on the survival of nevus or melanoma cells in collagen gel (data not shown). These findings implicated the presence of endogenous TGF-β1 in the cultures. To find out whether this was true, we prepared protein samples for Western blotting from cell lysates and serum-free conditioned medium. Fairly large quantities of TGF-β1-like immunoreactivity were found in melanocyte lysates but none in their 10×-concentrated conditioned medium (**Fig 6**). Comparable amounts of TGF-β immunoreactivity were also detected in nevus cell and melanoma lysates (data not shown). Samples of the collagen batch solutions were also analyzed to exclude the possibility that collagen contains TGF-β as an impurity. Western blotting failed to detect any TGF-β1-like immunoreactivity in the preparations (data not

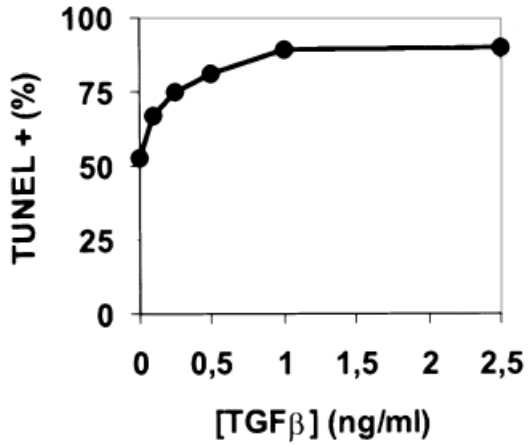

Figure 3. The effect of TGF-β1 on melanocyte apoptosis is dose dependent. Melanocytes were cultivated in collagen gel in the absence or presence of different TGF-β1 concentrations. The gels were fixed after 48 h and the cells were stained with bisbenzimide and by TUNEL. 300–500 nuclei were evaluated for TUNEL staining. The percentage of positive (apoptotic) cells is shown.

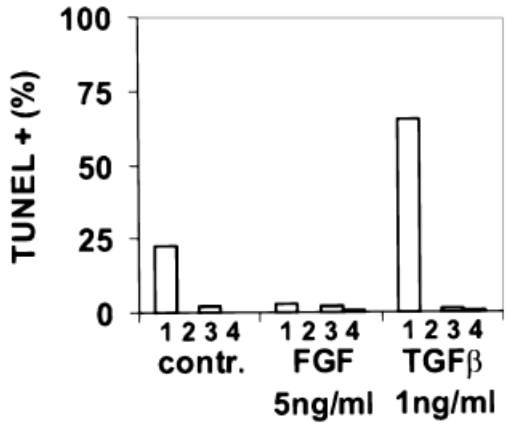

Figure 4. Nevus cells and melanoma cells are resistant to apoptosis caused by TGF-β1. Melanocytes (1), nevus cells (2), and ML793 (3) and G361 (4) melanoma cells were seeded between two layers of collagen gel. The gels were fixed after 48 h incubation in the presence or absence of FGF-2 (5 ng per ml) or TGF-β1 (1 ng per ml), and the cells were stained with bisbenzimide and by TUNEL. 300–500 cells were evaluated for TUNEL staining. The percentage of positive (apoptotic) cells is shown.

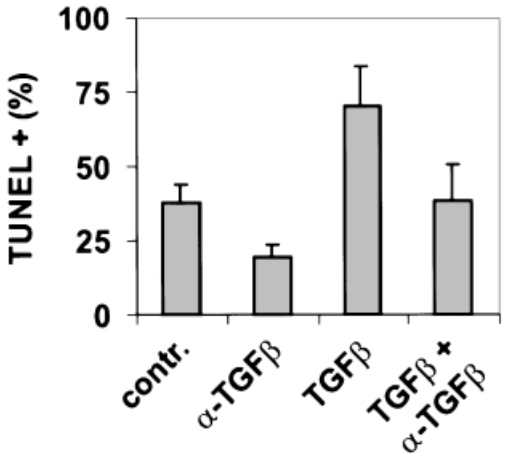

Figure 5. Neutralizing TGF-β1 antibodies inhibit the TGF-β-induced apoptosis of melanocytes in collagen and reduce also the background apoptosis that takes place in collagen in the absence of exogenous TGF-β1. Melanocytes were seeded into collagen gel in the absence or presence of TGF-β1 (0.5 ng per ml), neutralizing TGF-β1 antibodies (α-TGF-β; 0.5 μg per ml), or both (preincubated together 0.5 h at room temperature). Antibodies were added once after 24 h. Gels were fixed after 48 h and the cells were stained with bisbenzimide and by TUNEL. 300–500 nuclei were evaluated for TUNEL staining. The percentage of positive (apoptotic) cells is shown (*error bars:* ± SD in three experiments).

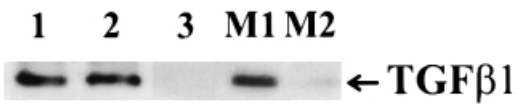

Figure 6. Western blot analysis indicates TGF-β-like immunoreactivity in melanocyte lysates. Melanocytes were seeded on a conventional cell culture plate or into collagen gel in the absence of serum. After 24 h their medium was collected, concentrated 10×, and mixed with a reducing sample buffer. Collagen was solubilized with collagenase and the cells were collected by centrifugation, washed with PBS, and lyzed with a lysis buffer. The cells grown on plates were washed and lyzed. The samples were electrophoresed in 12% SDS-PAGE, blotted on filter paper, and stained with TGF-β antibodies, appropriate secondary antibodies, and ECL reagents. Cell lysates from plate (*lane 1*) and collagen gel (*lane 2*), and the medium concentrate (*lane 3*) are presented. The arrow indicates the migration of 1 ng (M1) and 0.1 ng (M2) recombinant human TGF-β1 standards.

shown). Our Western blotting assay was typically able to detect 0.1 ng recombinant TGF-β1 per lane. As 50 μl concentrated medium was loaded, the theoretically lowest detectable concentration in conditioned medium was about 0.2 ng per ml.

Melanocytes produce latent TGF-β-like activity in both conventional and collagen culture Mv1Lu cells were used as indicator cells in a growth inhibition assay (see *Materials and Methods*) to enable more sensitive detection of TGF-β activity in conditioned medium. Medium was collected from cultures of melanocytes, nevus cells, and melanoma cells after 24 h incubation in the absence of serum in conventional culture or in collagen gel. Serum-free medium incubated with cell-free collagen alone was used as a control. The samples were heat treated (90°C, 1 min) to activate latent forms of TGF-β. The analyses indicated that melanocyte conditioned medium contained a detectable level of growth inhibitory activity that was almost totally in latent, heat-activatable form (**Fig 7*A***). The results obtained with nevus cell medium were almost identical. Under conventional culture conditions nevus cells seemed to have slightly less growth inhibitory activity in their medium compared to normal melanocytes. Melanoma cell conditioned medium contained considerably more TGF-β-like activity than the other cell types. No activity was released from the collagen preparations used (control). It is very unlikely that collagen contained latent forms of TGF-β, as the stock is strongly acidic before polymerization. The acidic pH is enough to convert TGF-β to active form (Lawrence *et al*, 1985). The growth inhibitory activity found in melanocyte, nevus cell, and melanoma medium could be partially blocked by neutralizing TGF-β1 antibodies (**Fig 7*B***).

DISCUSSION

Very few mitoses can be detected in normal melanocytes *in vivo* (Rieger *et al*, 1993). The reason for this is not well understood and is mainly considered to be lack of stimulation. Another characteristic feature of melanocytes is their strict adherence to the epidermal surface of the basement membrane next to basal keratinocytes. Previous studies have demonstrated that pigment cells isolated from the retina are very sensitive to the growth inhibitory effect of TGF-β. This has been suggested to be one of the *in vivo* mechanisms that keeps the retinal pigment cells in growth-arrested state (Hu *et al*, 1998). According to earlier work TGF-β also acts as an effective growth inhibitor for skin

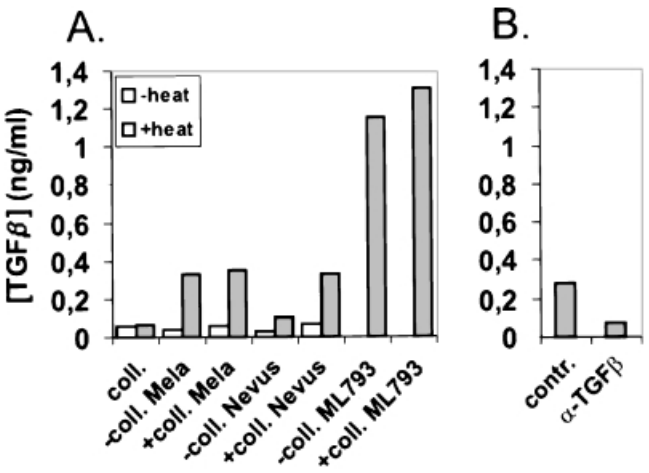

Figure 7. A sensitive bioassay indicates the presence of heat-activatable TGF-β-like activity in melanocyte, nevus cell, and melanoma medium. (*A*) Melanocytes (Mela), nevus cells (Nevus), and melanoma cells (ML793) were seeded on conventional cell culture plates or into collagen gel in the absence of serum. After 24 h their medium was collected. The medium was either used as such (– heat, *open bars*) or first heated to 90°C for 1 min to activate latent forms of TGF-β (+ heat, *gray bars*). Eighteen hours after transfer to Mv1Lu indicator cells 5-BrdU was added and incubated with the cells for 2 h. Active TGF-β effectively inhibits the replication of Mv1Lu cells and DNA incorporation of 5-BrdU. A standard curve was established using several concentrations of rhTGF-β1. The data are expressed as concentrations of rhTGF-β1 that produce comparable inhibition of Mv1Lu replication. The activity released from cell-free collagen (coll.) was analyzed as control. (*B*) The heat-treated melanocyte medium was incubated with control antibodies (contr.) or neutralizing TGF-β1 antibodies (α-TGF-β1) preceding transfer to indicator cells.

melanocytes (Rodeck *et al*, 1994). The connection between TGF-β and melanocyte apoptosis has not been established earlier. Here we show that TGF-β effectively inhibits the proliferation of stimulated skin melanocytes but has no effect on quiescent cells under conventional cell culture conditions. In the three-dimensional collagen culture, however, TGF-β induces rapid apoptosis of unstimulated melanocytes at picomolar concentrations. These findings suggest that TGF-β also participates in regulating the strict localization of melanocytes in the skin.

Recent studies utilizing dominant negative TGF-β receptors demonstrate that TGF-β is a negative growth regulator for keratinocytes *in vivo* (Wang *et al*, 1997; Amendt *et al*, 1998). The interstitial connective tissue of the dermis seems to act as a repository for TGF-β1 (Taipale *et al*, 1994; Karonen *et al*, 1997; Raghunath *et al*, 1998). Thus, TGF-β is present in all layers of the skin at concentrations capable of inducing apoptosis in melanocytes. To explain the survival of melanocytes in the epidermis, a role for contact-mediated positive signaling has been demonstrated by others (Scott and Liang, 1995; Hsu *et al*, 1996). Basal keratinocytes also express growth factors like FGF-2 and nerve growth factor, both of which are strong positive regulators of melanocyte growth and survival (Halaban *et al*, 1988; Pincelli and Yaar, 1997). Such strong local stimuli can presumably protect melanocytes from TGF-β-induced apoptosis. The opposing effects of FGF-2 and TGF-β on melanocyte apoptosis in collagen add a new dimension to melanocyte growth control. Positive signals keep the cells alive at the basement membrane zone and an effective negative growth regulator is ready to induce apoptosis in cells that lose their proper stimulation for survival.

Mv1Lu growth inhibition assay demonstrates that all the different melanocytic cell types tested secrete latent TGF-β-like activity to their growth medium. Active TGF-β can be detected only after heat treatment, however. Cultured cells typically secrete TGF-β in the latent form. The reduction of ''background'' apoptosis by neutralizing TGF-β antibodies indicates activation of the melanocyte-synthesized TGF-β in the collagen culture. Only part of the cell-secreted TGF-β is probably reached by the antibodies due to slow diffusion of immunoglobulin molecules, which may explain the only partial neutralizing effect of the added antibodies.

It is not known whether TGF-β is expressed by melanocytes *in vivo*. It is an intriguing possibility, as TGF-β could add to the control mechanisms by being activated under appropriate conditions, such as when melanocytes make contact with surrounding keratinocytes or with dermal fibroblasts. This kind of selective TGF-β activation takes place for example between endothelial cells and pericytes, most likely on the cell surface by proteolysis (Antonelli-Orlidge *et al*, 1989; Sato and Rifkin, 1989), and is thought to mediate the growth regulatory functions of the pericytes on vessel endothelium. It is important to notice that active TGF-β added to cultured melanocytes in the presence of positive survival signals only restricts their proliferation and does not induce apoptosis. This is analogous to the *in vivo* situation, where the melanocytes next to basement membrane form an almost nonproliferating cell population.

Nevus cells isolated from the dermis have a relatively high FGF-2 expression (Scott *et al*, 1991; Alanko *et al*, 1999). This seems to account for their survival in our collagen cultures and may also add to their resistance to TGF-β-mediated apoptosis. Nevus cells proliferate slowly in collagen gel without added FGF-2, and respond to active TGF-β by decreased proliferation rate but not by increased apoptosis. This is in accordance with the behavior of intradermal nevus cells *in vivo*, which mostly form a stable nongrowing tumor. It is tempting to speculate that it is the dermal TGF-β activation that counteracts their endogenous FGF-2 stimulation.

TGF-β is a candidate key factor to control the localization of melanocytes *in vivo*. As long as the cells remain sensitive to TGF-β-induced apoptosis and lack proper autocrine stimulation, they are not able to move outside the epidermis. The progression of melanocyte neoplasm would thus be restricted into the epidermis until the cells gain the ability to both resist apoptosis in the dermis and survive without the growth support provided by the basement membrane. An early melanoma *in situ* has the ability to survive in the upper layers of the epidermis but not in the dermis. It will be interesting to characterize the growth factor requirements and sensitivities of these particular potentially malignant cells. It has been clearly documented that FGF-2 expression alone does not convert melanocytes into malignant cells (Ramon y Cajal *et al*, 1991; Coleman and Lugo, 1998). As demonstrated by others also (Filmus and Kerbel, 1993; Rodeck *et al*, 1994; Krasagakis *et al*, 1999), malignant melanoma cells are unresponsive to negative regulation by TGF-β. Melanoma cells synthesize substantial amounts of several TGF-β isoforms, and their expression seems to increase during tumor progression (Van Belle *et al*, 1996). In other models tumor-cell-secreted TGF-β has been suggested to have important paracrine, possibly immunosuppressive, functions that favor tumor growth (Ruffini *et al*, 1993).

The mechanisms of TGF-β-induced apoptosis are not fully understood. Reactive oxygen species seem to play a role in mediating TGF-β-induced death of hepatocyte primary cultures (Sanchez *et al*, 1996), but transformed hepatoma cells have been reported to die through a different mechanism not involving oxygen radicals (Yamamoto *et al*, 1998). The studies conducted to reveal the effects of TGF-β on the members of the Bcl-2 family have not reached a conclusion. The effects seem to vary between different cell types and experimental settings.

Our results suggest that TGF-β is a central factor in the skin controlling not only the growth of keratinocytes but also the growth and localization of cells of the melanocytic lineage. As skin is an important organ that is rather easy to reach by topical and systemic treatments, in-depth knowledge of its normal biology and self-regulatory functions will eventually facilitate clinical use of growth factor preparations or even gene-targeted therapy.

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